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The Contribution of HPG axis-hormones and hormone replacement timing on cognition and associated signaling

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Jesal Shah was born in Gujarat, India in 1990. Raised in America, she had the opportunity to receive great education as well as to partake in many hobbies including gymnastics, swimming, band, and soccer. As a young adult, Jesal spent much of her time volunteering in hospitals and animal shelters, as she was an animal-lover as well as a fan of science. Aspiring to become a doctor, she majored in Biology at Case Western Reserve University and minored in Chemistry and Environmental Studies. Jesal is currently a CWRU Alumni and resides in Little Rock, AR where she will be attaining her Master's Degree at the University of Arkansas for Medical Sciences. She plans to go to Medical School to become a physician.

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ABSTRACT

Many women experiencing menopause show cognitive function deficits and have an increased risk of developing Alzheimer's Disease (AD). While estrogen (E2) has been shown to improve cognition and improve molecular signaling associated with learning and memory, these effects are lost if estrogen replacement is not started within a critical time period, close to menopause onset. During menopause the ovaries no longer produce estrogen. Thus, the negative feedback of estrogen onto gonadotropin production is lost, and gonadotropins such as Luteinizing Hormone (LH) increase substantially. The downregulation of LH (in the absence of E2) leads to cognitive improvements identical to those observed when E2 is administered right at the onset of menopause. This study attempts to find data on the changes in signaling proteins that are important for learning and memory. In order to test the hypothesis that cognitive changes after menopause are primarily driven by increases in LH not decreases in estrogen levels, mice were treated with LH regulators in the short-term, soon after menopausal onset, and in the long-term. They were then tested for levels of protein expression in the hippocampal area of the brain. This experiment attempts to determine whether the signaling molecules being studied are controlled by the menopausal state and other treatments. It determines whether estrogen's actions on these signaling cascades are independent of its ability to downregulate luteinizing hormone and whether the loss of effectiveness of E2 on cognition when treatment onset begins long after menopause is related to changes in these signaling cascades. The data reveals LA is better at downregulating LH than E2, especially in the long-term, and that the tested signaling molecules are affected by aging. The results are relevant in not only determining the effects of hormones and aging on the learning and memory process but also finding preventative measures for neural degenerative disorders such as Alzheimer's disease.

INTRODUCTION

Alzheimer's disease (AD) is an age-related, degenerative disorder that attacks nerve cells in the brain resulting in memory loss, decrease in thinking and language skills, and behavioral changes. Sex steroid hormones are regulators of neuron survival in multiple Central Nervous System regions and across a variety of circumstances ranging from normal development to neural injury [1]. The progressive decrease in sex steroids with age is an important factor for age-related Alzheimer's disease [1]. Hormones are not only a significant part of the endocrine system but

are also important in neural processes such as cognition. In women, the hormone estrogen plays a huge role in modulating the signaling cascades involved in cognition and regulating the signaling molecules involved in the processes of learning and memory. Abundant experimental, epidemiological and clinical evidence suggest that neural action estrogens can reduce the risk for AD [1]. This is evident in the increased risks of cognitive disorders that develop after menopause. In women, the age-related decrease in sex steroids is characterized by menopause via estrogen (E2). Women experiencing menopause have a tendency to show cognitive function deficits and are at risk of developing neurodegenerative diseases. In the hypothalamic-pituitary-gonadal (HPG) axis, the neurons within the hypothalamus release Gonadotropin-releasing Hormones (GnRH) to the anterior pituitary, which synthesizes and secretes the gonadotropin Luteinizing Hormone (LH). Normally in women, the ovaries produce estrogen which is part of the negative feedback loop to the hypothalamus in the brain. This results in an overall decrease in LH production. During menopause, the negative feedback of estrogen onto gonadotropin production is lost because estrogen is no longer being produced. As such, gonadotropins, for example luteinizing hormone (LH), increase substantially. The post-menopausal changes in E2 and LH levels have been associated with increased risk and development of AD and cognitive impairment [2, 3].

Conversely, the normalization of these hormones to physiological levels leads to cognitive improvement [4]. Hormone replacement therapy can artificially boost hormone levels, possibly prolonging life and reducing the incidence of dementia. Therefore if E2 replacement is administered at the onset of menopause, the treatment is effective; however, as the time interval between menopause onset and treatment increases, estrogen loses its ability to downregulate LH [3]. In the absence of E2, the drug Leuprolide Acetate (LA) can specifically downregulate elevations in the gonadotropin, LH, and can be used to differentiate estrogen from LH effects on neuronal function [5]. The gonadotropin releasing hormone antagonist can also improve cognitive function, and increase levels and phosphorylation of signaling molecules associated with the modulation of cognition [3]. This suggests that high LH, not low estrogen, is the primary driver of cognitive changes during menopause.

Elevations of serum LH occur during and after menopause. LH fluctuations have also been linked to cognitive deficits, AD predisposition, and decreased cognition-associated intracellular signaling [5]. Hormones such as estrogen are known to modulate cognition through a variety of intracellular cascades that are important in the formation and stabilization of long-term memories [6, 7]. Estrogen directly influences brain function through estrogen receptors located on neurons in multiple areas of the brain [14, 15]. The hormone also appears to have direct membrane-mediated effects on neurons. Its effects are both neuroprotective and neurotrophic [15]. Estrogen has been shown to protect isolated neurons in vitro from damage by amyloid protein, which is implicated in the pathogenesis of Alzheimer's disease [15]. At neuronal synapses, estrogen increases the concentration of neurotransmitters such as serotonin, dopamine, and norepinephrine [16]. It affects their release, reuptake, and enzymatic inactivation. It also increases the number of receptors for these neurotransmitters [15]. Synaptophysin and synapsin are both important molecules in the regulation of synaptic release and are both associated with improvements in memory function [10, 11]. Synaptophysin (SYP) is a calcium-binding glycoprotein found in the membranes of neurotransmitter-containing presynaptic vesicles, and it is believed to modulate the ef-

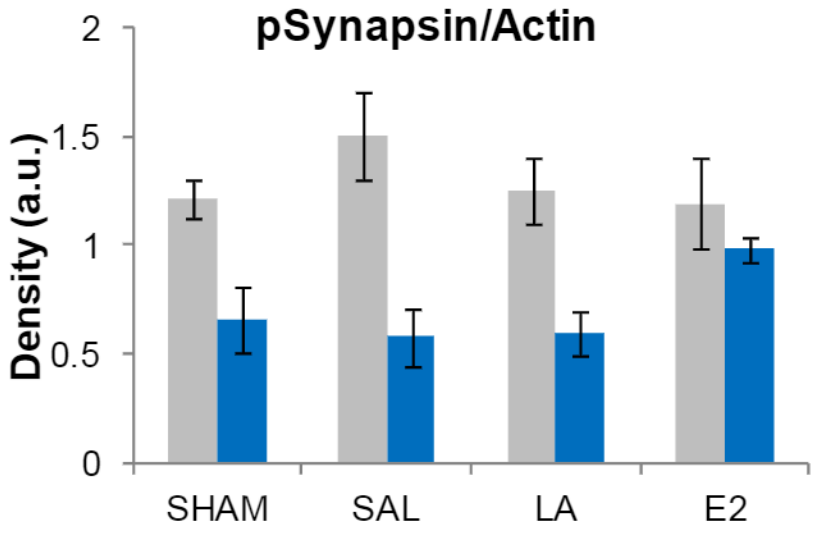

Figure 1. Protein expression of phosphorylated synapsin. Short-term treatment shown in grey and long in blue. There is no effect of ovariectomy or any of the treatments when started short term after ovariectomy. E2 replacement started a long interval after ovariectomy significantly upregulates the levels of pSynapsin compared to all other groups.

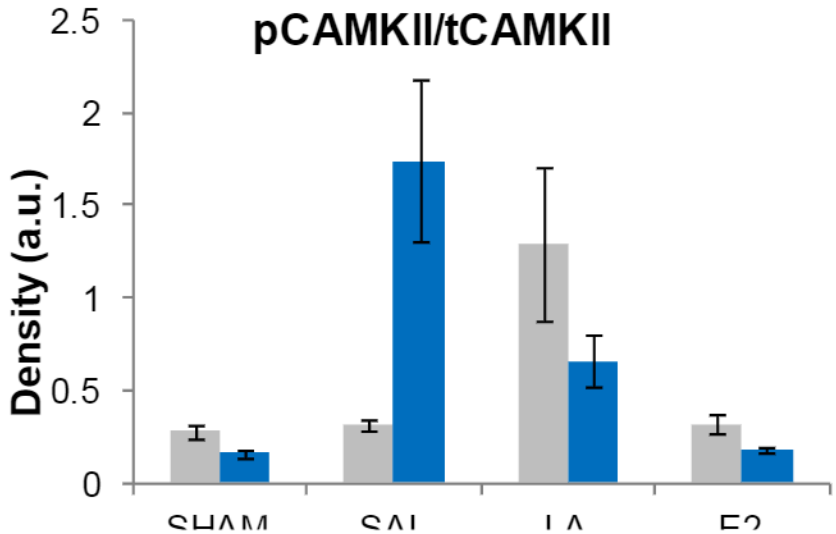

Figure 2. Protein expression of phosphorylated CAMKII. Short-term treatment shown in grey and long in blue. There is no effect of ovariectomy on pCAMKII expression; however, LA treatment leads to a significant upregulation compared to all treatments. pCAMKII is significantly upregulated by OVX when measured 6 months after the procedure compared to all groups. LA treatment when started long-term is significantly downregulated compared to short-term and long-term OVX but still significantly elevated compared to SHAM and E2 groups.

ficiency of synaptic vesicle cycles. Normally, synaptophysin decreases with age in the hippocampus and various cortical regions. Synapsin is an abundant brain protein essential for regulating neurotransmitter release. Phosphorylation of synapsin by PKA or CaM Kinase I inhibits its binding to phospholipids and dissociates synapsin from synaptic vesicles [17]. Synapsin I is an exclusively presynaptic protein, so its deletion can block the enhancements of learning, presynaptic plasticity, and Long Term Potentiation (LTP) [11].

Memory is created by the persistent modification of strength of synapses. One such modification is LTP which occurs in the hippocampus. LTP has associative properties that match the process of learning. Once it is triggered by the rise in postsynaptic calcium, modification can be maintained for up to an hour [8]. In order to facilitate both LTP and the formation of new memories, glutamate receptor 1 (GluR1), an important subunit of the AMPA receptor, must be phosphorylated at amino acid Ser845, which leads to the mobilization of the AMPA receptor from the post-synaptic density to the synapse [6, 9, 18]. Signaling events, such as the autophosphorylation of calcium-calmodulin kinase II (CAMKII), lead to the phosphorylation of GluR1 [9]. The maintenance of AMPA receptors is due to the ability of CaMKII to maintain its activity for long periods of time after its initial activation by calcium. CaMKII is a critical protein in LTP because it has special properties which make it exhibit persistent changes.

Women experience both elevations in LH and declines in estrogen during and after menopause. Similarly, after ovariectomy (OVX), rodents also have elevations in LH and declines in estrogen providing a good model to study hypothalamic-pituitary-gonadal-axis hormone-cognition interactions [12] as well as the learning and memory signaling cascades. It is unknown if the changes in signaling cascades are related to 1) whether the signaling molecules studied are modulated by menopausal state and our treatments, 2) whether E2 actions on these signaling cascades are independent of its ability to downregulate LH, and 3) whether loss of effectiveness of E2 on cognition when treatment onset begins long after menopause. Therefore, it is the goal of this project is to study the changes in the signaling proteins important for learning and memory to determine such relationships.

MATERIALS AND METHODS

Animals

Female, 8 month-old C57/BL6 mice were ovariectomized (OVX) and treated with saline (SAL), Leuprolide Acetate (LA) (7.5 mg/kg SC) (for the downregulation of LH) and E2 (5ug SC) for 3 months. The SAL treatment is also known as OVX and will be used interchangeably. This treatment emulated the symptoms of menopause. For the short-term treatments, the drugs administered immediately after surgery from months 1 to 3. For the long-term treatments, drugs were administered 4 months after surgery to determine effects of treatment timing. The SHAM mice served as a control since they did not experience menopausal conditions.

Tissue processing and Western Blots

For western blot analysis, hippocampus tissue was homogenized, and protein levels were determined for approximately 16 samples. The hippocampus was chosen for testing because it is the center of the brain associated with memory and cognition. 20ug aliquots of tissue were loaded and separated by electrophoresis on 8% acrylamide gels. Primary antibodies of both phospho- and total CaM Kinase II (Promega), synaptophysin (Invitrogen), and phospho-Synapsin (Calbiochem) were used in dilutions of

1:5000, 1:1000, and1:200 respectively. Gels were then transferred to PVDF membranes, probed with rabbit antibody, and visualized using a chemiluminescence-based detection (Denville). The blots were compared to the loading-control protein actin, which is expressed at a constant level regardless of the treatment applied to the original organism.

Quantification

Western blots of both the tested proteins as well as the actin were quantified by densiometric measurements using ImageJ (NIH) data analyzed by ANOVA and post-hoc comparisons.

RESULTS

Overall, our data reveals trends in protein expression as the term of the treatment increases. Phospho-synapsin expression decreases with age regardless of the treatment. Phospho-CAMKII expression as opposed to total CAMKII expression varies with regards to treatment, while synaptophysin expression increases with age in each treatment group.

Phospho-synapsin is not affected by ovariectomyor by treatments in the short term after ovariectomy, but it is reduced in the long term with each treatment. Interestingly, the reduction in phospho-synapsin expression from E2 replacement had a smaller difference compared to the SHAM mice and the other treatments (Figure 1).

The trend of decreased phospho-CAMKII expression with increasing age is not as defined as with the other proteins because with the SAL treatment there is an over-expression of the protein long after the onset of menopause- in particular 6 months after; however the autophosphorylation of CAMKII is not affected within 3 months from the OVX. LA increased levels of pCAMKII significantly at both time-points yet, levels in LA treated animals were significantly lower to those of OVX animals in the long-term group (Figure 2).

The trend in synaptophysin expression is age-related upregulation independent of the groups being treated. The protein expression is not altered by OVX or any of the treatments. When LA treatment is started 4 months post-OVX, the downregulation of LH significantly increased synaptophysin expression beyond age-associated levels. However, the E2 treatment started 4 months after post-OVX shows a smaller increase in protein expression when compared to the other treatments (Figure 3).

CONCLUSION AND DISCUSSION

Based on the results, the hypothesis that cognitive changes after menopause are primarily driven by increases in LH, not decreases in estrogen levels, is partially proven because the effects of LA and E2 are very similar in protein expression immediately post-OVX, but different long after OVX. This tells us that E2 is not as effective as LA when applied in the long-term, hence proving that cognitive changes post menopause are driven by increases in LH.

In addition, the data discloses that the tested signaling molecules are not considerably modified during menopause at least within the first 3 months. This suggests that cognitive impairment observed by OVX is unlikely to be driven through these cascades and are affected by some other cascades. Our results also show that synaptic transmission associated proteins show age-related effects in all our groups. For example, synaptophysin decreases with age in the hippocampus and various cortical regions [7]. Previously published data has shown that aging can lead to the incorrect regulation of many proteins involved in neuronal signaling that may be associated with age-re-

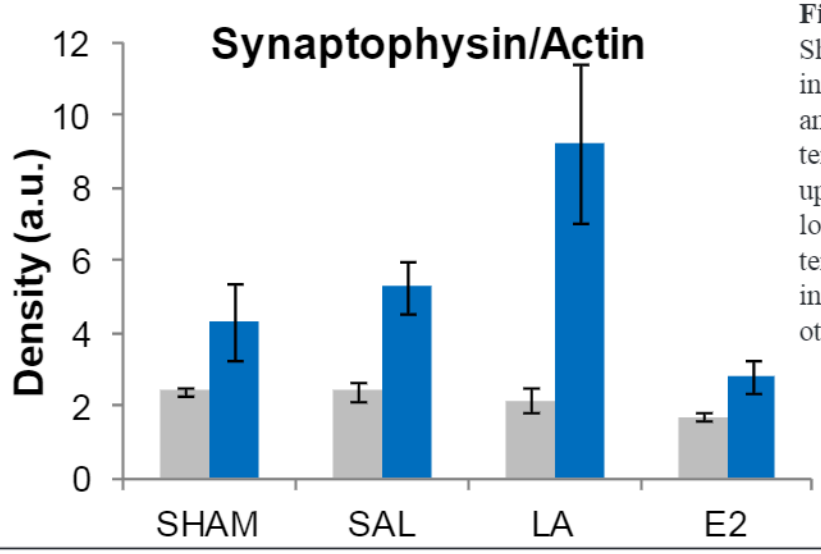

Figure 3. Protein expression of synaptophysin. Short-term treatment shown in grey and long in blue. There is no effect of ovariectomy or any of the treatments when administered short-term after OVX. Synaptophysin expression is upregulated by age in all conditions (short vs. long). LA treatment when administered long-term after OVX leads to a significant increase in synaptophysin expression compared to all other groups.

lated diseases [13]. This makes sense because protein levels can be regulated by hormonal control, but with time, incorrect hormone levels can result in the dysfunction of proteins involved in synaptic transmission. Dysfunction in transmission can lead to a decrease in neurotransmitter release.

In another set of results, age-related downregulation of phospho-synapsin is prevented by E2 replacement when treatment is started long after OVX. The significance of these findings is unclear given that E2 replacement is not effective at improving cognition at this later interval. On the other hand, LA increases synaptophysin expression in our long-term group. It is unlikely that synaptophysin is a major modulator of cognition after menopause based on our data; instead, it might modulate the efficiency of synaptic vesicle cycles. However, the additional increases produced by LA could take part in cognitive protection. This could be due to LA being more effective than E2 in downregulating LH in the long term.

Lastly, while pCAMKII is not substantially changed by OVX at 3 months, it is significantly increased at 7 months post OVX. This could be in response to the decrease in levels of neurotransmitter release post-OVX in the long term due to synapsin. The increase in pCAMKII allows more AMPA receptors to reach the synapse to accept the few neurotransmitters released. On the other hand, LA treatment increases pCAMKII when treatment is started immediately after and long-term compared to SHAM, but the magnitude of increase is significantly lower than that of OVX (long-term). This is reasonable because LA downregulates LH creating effects similar to SHAM and E2.

It is challenging to isolate these signaling cascades to guarantee that other factors are not interfering with the processes. For example, calcium plays a huge role in cell signaling and transduction. CAMKII autophosphorylation is specific to intracellular calcium release, which is known to both improve cognition and lead to cytotoxicity. Differences in the magnitude of pCAMK in this study may therefore reflect positive and noxious increases in Ca++ release. The next steps would possibly be to test more samples, test other proteins in the signaling cascade to confirm the process and reasons why the levels go as the results show, or to somehow isolate these signaling cascades and see their effects purely from the treatments administered.

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